In Vitro Cell. Dev. Biol.—Plant 36:13–20, January–February 2000

1054-5476/00 $10.00+0.00

EFFECT OF THE CAULIFLOWER MOSAIC VIRUS 35S, ACTIN, AND UBIQUITIN PROMOTERS ON *UIDA* EXPRESSION FROM A *BAR-UIDA* FUSION GENE IN TRANSGENIC *GLADIOLUS* PLANTS

KATHRYN KAMO,[1]* ALAN BLOWERS[2] AND DAVID MCELROY[3]

[1]*Floral and Nursery Plants, B-010A Room 238 BARC West, U.S. Department of Agriculture National Arboretum, Beltsville, MD 20705-2350,* [2]*Sanford Scientific, Inc., 877 Marshall Drive, Waterloo, NY 13165,* [3]*DEKALB Genetics Corp., 62 Maritime Drive, Mystic, CT 06355*

(Received 11 February 1999; accepted 14 September 1999; editor P. Ozias-Akins)

SUMMARY

Tissue-specific patterns and levels of gene expression were characterized in transgenic *Gladiolus* plants that contained the phosphinothricin acetyltransferase (*bar*)-β-glucuronidase (*uidA*) fusion gene under transcriptional control of the promoter from either the cauliflower mosaic virus 35S (CaMV 35S), duplicated CaMV 35S (2 × CaMV 35S), rice actin (*Act1*), or *Arabidopsis* ubiquitin (*UBQ3)* promoters. The *bar* gene confers resistance to phosphinothricin (PPT)-containing herbicides and allowed selection of transgenic cells. The β-glucuronidase gene encoded by the *uidA* locus of *E. coli* functioned as a reporter gene. Maximum levels of β-glucuronidase (GUS) activity in leaves were 173, 112, 50, and 10 nmoles 4-methylumbelliferone h^{-1} mg^{-1} protein for transgenic plants with the *bar-uidA* fusion gene under the control of the CaMV 35S, 2 × CaMV 35S, *UBQ3*, and *Act1* promoters, respectively. There was frequently considerable variability in GUS activity between the leaves of a single plant, and levels of *uidA* expression varied between independently transformed plants for each promoter. Callus derived from transgenic plants showed much less variation in GUS expression than leaves. The mean level of GUS activity was significantly higher (over 3×) for transgenic lines of callus containing the CaMV 35S as compared to the *UBQ3* promoter, and this confirmed the higher (2×) level of GUS activity in leaves of plants with the CaMV 35S promoter as compared to the *UBQ3* promoter. Tissue-specific patterns of *uidA* expression were determined by histochemical staining. Leaves 5–6 cm long from plants with any of the four promoters tested exhibited *uidA* expression primarily in the vasculature. Under all four promoters *uidA* was expressed more frequently in root tips as compared to leaves.

Key words: GUS; monocotyledonous bulbs; *Gladiolus*; transformation; transgenic.

INTRODUCTION

The regulation of transgene expression is critical if genetic engineering is to be successfully applied for commercial purposes. A transgene of interest must be expressed in a specific plant tissue and at a level suitable for its intended purpose. Regulation of gene expression in monocotyledonous plants has primarily involved studies of the agronomically important cereal crops such as maize and rice. The similarities in tissue-specific or developmental gene expression that occurred among a number of transgenic cereals and resulted from a specific promoter suggested that the control of gene expression by promoters was similar amongst the cereals (Shimamoto, 1995).

Transient *uidA* expression studies (Schledzewski and Mendel, 1994; Wilmink et al., 1995) have shown that most of the cereal monocots exhibit significantly higher levels of GUS activity with monocot-derived promoters such as from the *Act1* (rice actin) gene (McElroy et al., 1991; Zhang et al., 1991) or the *Ubi1* (maize ubiquitin) gene (Christensen et al., 1992) as compared to the CaMV 35S (cauliflower mosaic virus) promoter (Odell et al., 1985). In comparison, studies of a few noncereal monocots such as *Lolium*, certain members of the Liliaceae, and *Gladiolus*, have revealed that the noncereal monocots showed lower levels of transient *uidA* expression with the *Act1* and *Ubi1* promoters than with the CaMV 35S promoter (Kamo et al., 1995a; Wilmink et al., 1995).

This apparent difference in the regulation of gene expression exhibited by some noncereal monocots has only been studied in transient expression systems. In this study transgenic plants of *Gladiolus* were developed to characterize the tissue-specific patterns and levels of *uidA* expression from either dicot or cereal promoters in a noncereal monocot. The promoters utilized in developing transgenic *Gladiolus* plants were the *Act1*, CaMV 35S, duplicated CaMV 35S, and *Arabidopsis UBQ3* (Norris et al., 1993) in conjunction with a *bar-uidA* fusion gene. The *bar-uidA* fusion gene was selected for this study because it was anticipated that fusion of the *bar* gene with *uidA* would stabilize the *uidA* transcripts, resulting in an increased number of cell lines recovered that expressed *uidA*, and *uidA* expression would continue for a longer period of time with a fusion gene as compared to *uidA* expression resulting from co-transformation. Others (Spencer et al.,

*Author to whom correspondence should be addressed: Email kkamo@asrr.arsusda.gov

1992; Register et al., 1994) have reported the loss of *uidA* expression with time, and it was hoped that this could be avoided in this study.

Materials and Methods

Plant material. Callus was initiated from *in vitro*-grown cormel slices of *Gladiolus* cultivar 'Jenny Lee.' Cormel slices were cultured in the dark at 26°C on Murashige and Skoog's (MS) basal salts medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose, 0.2 mg l^{-1} Gelrite (Sigma Chemical Co., St. Louis, MO) and the following in mg l^{-1}: glycine, 1.0; thiamine, 1.0; pyridoxine, 0.5; nicotinic acid, 0.5 and supplemented with either 2.2 μM 2,4-dichlorophenoxyacetic acid (2,4-D) or 9.3 μM 3,6-dichloro-2-methoxybenzoic acid (dicamba) for callus induction. The medium was adjusted to pH 5.8 and then autoclaved for 20 min at 121°C, 18 psi. Callus induction and multiplication occurred on the same medium and required 6 mo. Suspension cultures were initiated from the callus, and were grown in 20 ml of the same medium as used for callus growth with the omission of Gelrite. Suspension cells were subcultured every week at a 1:1 dilution to fresh medium. Cells were grown in the dark at 26°C at 120 rpm on a gyratory shaker.

Plasmid DNA. Plasmid DNA was isolated from *E. coli* strain DH5α using alkaline lysis followed by purification from a cesium chloride gradient (Maniatis et al., 1982). Transgenic plants of *Gladiolus* were developed for the *Act1* promoter using pDM343, the CaMV 35S promoter using pDM327, the duplicated CaMV 35S promoter (derived from pBI410, received from William Crosby, Saskatchewan, Canada; Russell et al., 1992) using psan18, and the *UBQ3* promoter using psan152 (Norris et al., 1993). All of these constructs contained the 2.5 kb *bar-uidA* fusion gene derived from pDM327 following digestion with *Sst* I (Fig. 1). The *bar* coding region minus the translation termination codon was isolated as a 0.6 kb *Eco*RI-*Bgl*II restriction fragment from pIJ4104 (Murakami et al., 1986), and this was cloned between the *Eco*RI and *Bam*HI restriction sites of the *uidA-nos*-containing construct pDM201.3 (McElroy et al., 1995) to create the *bar-uidA-nos* translational fusion construct pDM323. The CaMV 35S promoter was isolated as an 0.8 kb *Hind*III-*Eco*RI restriction fragment from the 35S-*bar-nos*-containing construct pDM307 (Cao et al., 1992), and this was cloned between the *Hind*III and *Eco*RI restriction sites of pDM323 to create the 35S-*bar-uidA-nos* translational fusion construct pDM327.

Transgenic *Gladiolus* plants containing *uidA* under either the *Ubi3* or *Ubi7* promoter (Garbarino and Belknap 1994; Garbarino et al., 1995) were developed by co-bombardment with p35SAc that contains the phosphinothricin acetyltransferase (PAT) gene (P. Eckes, Hoechst Roussell Co., Somerville, NJ) under the CaMV 35S promoter. Both *bar* and *pat* genes encode a phosphinothricin acetyltransferase of comparable molecular weight and have a similar affinity for L-phosphinothricin, although each gene was isolated from a different species of *Streptomyces* (Wehrmann et al., 1996). *Pat* had been used previously to recover transformants of *Gladiolus* (Kamo et al., 1995a).

Particle gun bombardment. The PDS-1000/He system (BioRad, Richmond, CA) that consists of a helium-driven particle accelerator with macrocarrier was used for particle delivery to suspension cells that were 1 wk old after subculturing at a 1:1 dilution. The particle gun was set up with a 1-cm gap and flying membrane distance. The target distance from the stopping screen was 12 cm, and cells were bombarded at 8.3 MPa (1200 psi) once per plate. Gold (1 μm) particles were coated with plasmid DNA according to the method of Sanford et al. (1993). The concentration of DNA used was either 2 μg DNA per μg gold for the fusion gene constructs or 1 μg of p35SAc mixed with 1 μg of either the *Ubi3* or *Ubi7* promoter DNA construct per μg gold for co-bombardment.

Suspension cell selection. One week after particle gun bombardment, cells were transferred to Gelrite-solidified MS basal salts medium supplemented with 2.2 μM 2,4-D and either 1 or 3 mg l^{-1} bialaphos, a tripeptide containing phosphinothricin (PPT; Meiji Seika Kaisha, Tokyo) or 6 mg l^{-1} PPT (Hoechst Roussell Co., Somerville, NJ). Every 3–4 wk the cells were transferred to fresh media with the same selective agent. Cells were grown at 26°C in the dark. After approximately 3–6 mo. of selection, the majority of cells appeared dead. Remaining calluses were transferred to regeneration medium and grown at 26°C under a 16-h light photoperiod. Regeneration medium consisted of MS basal salts medium supplemented with 9.3 μM kinetin and either 1 or 3 mg l^{-1} bialaphos. Regenerated shoots that were approximately 1 cm long were transferred to MS basal salts medium that contained no hormones and no selective agent. Roots rapidly formed on the shoots after 1–2 wk. The small plants that expressed *uidA* by histochemical staining were selected for further analysis.

GUS evaluation. Histochemical and fluorimetric determinations of GUS enzyme activity and *uidA* gene expression were performed according to the method of Jefferson et al. (1987). Histochemical staining of plant cells consisted of placing the plant material in fixative (0.3% formaldehyde in 10 m*M* 2-*N*-morpholinoethanesulfonic acid (MES), pH 5.6, 0.3 *M* mannitol) for 45 min followed by three washes, each 15 min, in 50 m*M* sodium phosphate monobasic (NaH_2PO_4), pH 7.0. The fixative and phosphate buffer washes were allowed to penetrate the tissues by vacuum infiltration for 5 min. Tissues were then incubated for 16 h at 37°C in a 50 m*M* NaH_2PO_4, pH 7.0 solution containing 1 m*M* 5-bromo-3-chloro-3-indolyl-β-D-glucuronic acid that had been dissolved in a minimal amount of dimethyl sulfoxide.

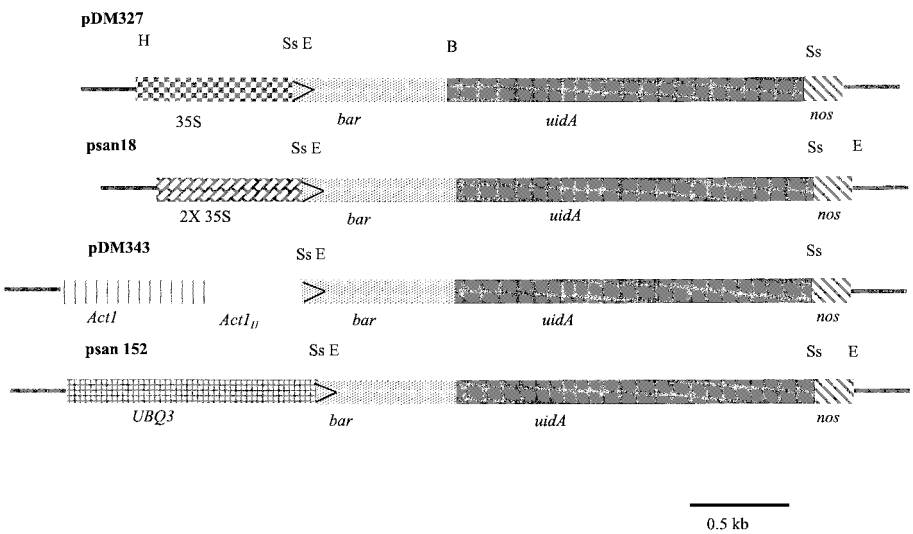

FIG. 1. Diagram of the *bar-uidA* fusion genes. Top to bottom: 35S RNA promoter from the cauliflower mosaic virus; the duplicated 35S RNA promoter; the rice actin 5′ region that contains the promoter and the first intron; and the *Arabidopsis UBQ3* promoter. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Ss, *Sst*I.

Leaf tissues were then destained by incubating for 16 h at 26°C in 70% ethanol. Either leaf and root tissues that were approximately 1 cm long were excised from plants, or whole intact plants were stained every 4 mo. for 1 yr following regeneration. Each promoter was represented by 15–23 transformed plant lines for histochemical staining.

The specific activity of GUS expression was measured by fluorimetric determination of 4-methylumbelliferone (4-MU) production (Jefferson et al., 1987). Extracts from transgenics were prepared by grinding approximately 300 mg fresh weight of plant leaves or 100 mg fresh weight of callus using a mortar and pestle in 500 μl extraction buffer (50 m*M* NaH_2PO_4, pH 7.0, 10 m*M* disodium ethylenediaminetetraacetic acid (EDTA), pH 8.0, 0.1% Triton X-100, 0.1% sarkosyl, and 10 m*M* β-mercaptoethanol) and sonicating the homogenate for 3 min. Extracts were centrifuged at 10,000 × *g*, 4°C for 5 min, and an aliquot of the supernatant was added to the assay buffer (1 m*M* methylumbelliferyl-β-D-glucuronide) for incubation. After 15, 30, and 60 min incubation at 37°C, an aliquot of the incubation mixture was added to 0.2 *M* sodium carbonate. The fluorescence was measured with a Perkin–Elmer spectrofluorometer LS-3 set at 365 nm for excitation and 455 nm for emission. Protein concentration of the plant extracts was measured by using the bicinchoninic (BCA) protein assay reagent (Pierce Co., Rockford, IL) according to the manufacturer's instructions.

The mean specific activity of β-glucuronidase for each independently transformed plant was determined from three plants grown in separate Magenta vessels (Magenta Corp., Chicago, IL) *in vitro* or from three callus pieces each grown in separate petri plates. Several leaves (three to four) were harvested from each fused cluster of *in vitro*-grown plants 3 wk after the plants had been subcultured into MS basal salts medium lacking hormones. Callus had been initiated from the base of *in vitro*-grown plants after 5–6 mo. of culture on MS basal salts medium supplemented with 2.2 μ*M* 2,4-D. The callus was separated from the plant and maintained on MS basal salts medium supplemented with 2.2 μ*M* 2,4-D. Callus was collected 2 wk after subculturing for analysis. Nontransformed *Gladiolus* 'Jenny Lee' tissues and callus were used as the negative controls for both fluorimetric and histochemical determinations.

Genomic DNA analysis. Southern hybridization was performed using genomic DNA isolated from suspension cells derived from *in vitro*-grown plants according to the method of Dellaporta et al. (1983). DNA (10 μg) digested with either *Sma*I or *Sst*I was subjected to electrophoresis in a 0.7% agarose gel in TBE buffer (89 m*M* Tris, 89 m*M* boric acid, 2 m*M* EDTA, pH 8.0) at 75 V for approximately 4 h. Capillary movement was employed to transfer the DNA from the agarose gel to a Nytran nylon membrane (Schleicher and Schuell, Keene, NY) (Maniatis et al., 1982).

The 2.7 kb probe consisting of *bar-uidA-nos* was derived from psan152 digested with *Eco*RI followed by gel purification using Prepagene according to the manufacturer's instructions (BioRad, Richmond, CA). The probe was labeled by random priming and incorporation of [α-^{32}P] dCTP using the Megaprime kit (Amersham, Arlington Heights, IL). DNA blots were incubated in prehybridization buffer [6 × sodium chloride/sodium citrate (SSC), 0.5% sodium dodecyl sulfate (SDS), 5 × Denhardt's solution, 100 μg ml^{-1} denatured, salmon sperm DNA] for 2 h at 42°C followed by hybridization buffer (6 × SSC, 0.01 *M* EDTA, 5 × Denhardt's solution, 0.5% SDS, 100 μg ml^{-1} denatured salmon sperm DNA) with a probe at 60°C for 16 h (Maniatis et al., 1982). The blot was then washed three times in 6 × SSC, 0.1% SDS, 5 min each wash at 26°C, followed by two washes in 1 × SSC, 1% SDS, 1 min each wash at 65°C, and lastly two washes in 2 × SSC, 5 min each wash at 26°C. Blots were exposed to X-ray film at −70°C for 5 to 14 d with an intensifying screen.

RESULTS

Recovery of transgenic Gladiolus *plants.* The largest number (109) of putatively transformed plants was recovered from 40 plates of suspension cells bombarded with either pDM327 or psan18 that contained *bar-uidA* under either the CaMV 35S or duplicated CaMV 35S promoter, respectively. In comparison, 23 putatively transformed plants were recovered from 21 plates of cells bombarded with *bar-uidA* under the *Act1* promoter, and 27 plants were recovered from 21 plates bombarded with psan152 that contained the *UBQ3* promoter. Over 90% of the putatively transformed plants containing either the CaMV 35S, *Act1*, or *UBQ3* promoters showed *uidA* expression 6 mo. following their regeneration. Only 43% of putatively transformed plants co-transformed with *uidA* under either the *Ubi3* or *Ubi7* promoter showed *uidA* expression. All transgenic plants were initially selected based upon PPT resistance, and those plants that showed *uidA* expression were further analyzed.

DNA gel blot analysis verified the presence of the various plasmid DNAs in the genomic DNA of *Gladiolus* plants (Fig. 2). The expected 2.5 kb *bar-uidA* fusion gene was observed in transgenic plants containing *uidA* under either the CaMV 35S, *Act1* (Fig. 2 A, lanes 5 and 7), the duplicated CaMV 35S or *UBQ3* promoters (Fig. 2 B, lanes 5 and 7, respectively). The expected 5.7 kb band (size based upon digestion of psan18 plasmid DNA) was not seen from genomic DNA of plants containing the duplicated CaMV 35S promoter following digestion with *Sma*I (Fig. 2 B, lane 6), although this band, as well as a 3.0 kb band representing a rearrangement, were seen following a longer exposure of the autoradiogram (data not shown).

Levels of GUS expression. There was great variation in *uidA* expression between independent transformants of *Gladiolus* containing the CaMV 35S promoter as has been reported for maize. Six individual leaves of *Gladiolus* showed a 12.3-fold variation in level of GUS expression although the leaves were derived from a single, clonal line of *Gladiolus* plants that contained the CaMV 35S promoter, and the plants were grown *in vitro* within a single Magenta jar. The large variation in GUS expression between leaves of transgenic *Gladiolus* plants and the small number of plants analyzed resulted in statistically nonsignificant differences between plants containing the four promoters (Table 1). The data on levels of GUS expression for the four promoters primarily indicated that a plant containing the CaMV 35S promoter will have the capacity to express a higher level of GUS (173 nmoles 4-MU h^{-1} mg^{-1} protein) more frequently than plants with either the *Act1* or *UBQ3* promoters (Fig. 3 C–F). The highest level of GUS activity attained by one of five transgenic plants containing the *Act1* promoter was 10 nmoles 4-MU h^{-1} mg^{-1} protein as compared to a

TABLE 1

EFFECT OF PROMOTER ON LEVELS OF GUS EXPRESSION IN LEAVES OF TRANSGENIC *GLADIOLUS* PLANTS GROWN *IN VITRO*. THE SPECIFIC ACTIVITY OF β-GLUCURONIDASE (MEAN ± STANDARD ERROR) FOR EACH PROMOTER IS CALCULATED FROM FOUR TO EIGHT INDEPENDENT TRANSFORMANTS EACH SAMPLED THREE TIMES. ONLY PLANTS THAT EXPRESSED GUS WERE INCLUDED

		nmoles 4-MU h^{-1} mg^{-1} protein		
Promoter	Source	Highest level	Mean[a]	Relative GUS expression[b]
35S	CaMV	173	37.9 ± 14.5z	1.0
2 × 35S	CaMV	112	31.6 ± 18.1z	0.8
Act1	Rice	10	4.5 ± 0.8z	0.2
UBQ3	*Arabidopsis*	50	15.6 ± 4.9z	0.5

[a]Values followed by the same letter are not significantly different at $P \leq 0.05$ according to Kruskal–Wallis one-way analysis of variance.

[b]Values represent relative GUS expression where 35S-GUS is set at 1.0.

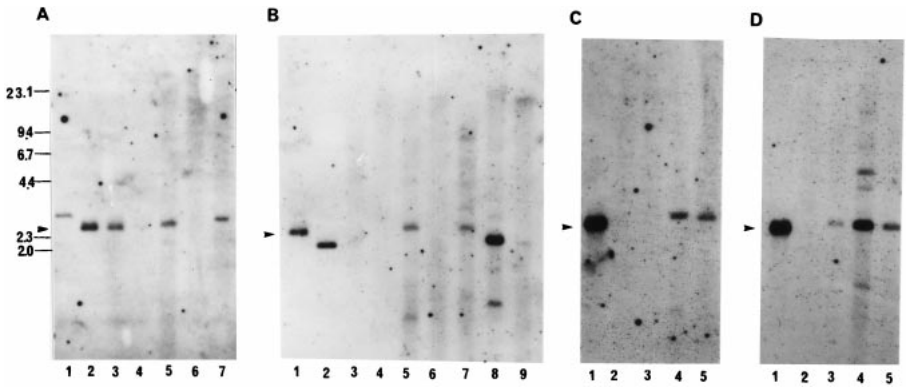

FIG. 2. DNA blot of genomic DNA isolated from suspension cells of *Gladiolus* and probed with psan152 digested with *Eco*RI which releases a 2.7 kb *bar-uidA-nos* insert. The 2.5 kb *bar-uidA* insert is shown by the arrow in A, B, C and D. A. *Gladiolus* transformed with *bar-uidA* under either the CaMV 35S (pDM327) or *Act1* (pDM343) promoters. *Lane* 1: psan152 digested with *Eco*RI which released 25 pg of a 2.7 kb *bar-uidA-nos* insert. *Lanes* 2 and 4: pDM327 digested with *Sst*I which released 50, 25, 5 pg, respectively, of a 2.5 kb *bar-uidA* insert. *Lanes* 5 and 7 each contain 10 μg genomic DNA digested with *Sst*I from *Gladiolus* transformed with either pDM343 or pDM327, respectively. *Lane* 6 is nontransformed *Gladiolus*. Size markers (kb) are shown on the left. B. *Gladiolus* transformed with *uidA* under either the duplicated CaMV 35S (psan18), *UBQ3* (psan152), *Ubi3*, or *Ubi7* promoters. *Lane 1*: pDM327 digested with *Sst*I to release 50 pg of a 2.5 kb *bar-uidA* insert. *Lane* 2: *Ubi3* digested with *Sma*I which released 50 pg of a 2.2 kb *uidA-nos* insert. *Lanes* 3 and 4: Genomic DNA from nontransformed *Gladiolus* digested with either *Sst*I or *Sma*I, respectively. *Lanes* 5 and 6: Genomic DNA from a *Gladiolus* plant transformed with psan18, digested with either *Sst*I (*lane* 5) or *Sma*I (*lane* 6). *Lane* 7: Genomic DNA from a *Gladiolus* plant transformed with psan152, digested with *Sst*I. *Lanes* 8 and 9: Genomic DNA from a *Gladiolus* plant transformed with either *Ubi3* (*lane* 8) or *Ubi7* (*lane* 9), digested with *Sma*I. C and D. *Gladiolus* lines transformed with *bar-uidA* under the CaMV 35S promoter with leaves that expressed the following levels of GUS activity (nmoles 4-MU h^{-1} mg^{-1} protein): 173 and 21 (C, *lanes* 4 and 5), 28, 1, 1 (D, *lanes* 3–5), respectively. Genomic DNA from transformed and nontransformed plants (C and D, *lane* 2) was digested with *Sst*I. *Lane* 1: pDM327 digested with *Sst*I released 50 pg of a 2.5 kb *bar-uidA* insert.

maximum level of 50 nmoles 4-MU h^{-1} mg^{-1} protein for one of five plants containing the *UBQ3* promoter (Fig. 3 D, F).

GUS activity of callus derived from a transgenic plant showed less variation in GUS than leaves from the same plant; however, the levels in callus were much higher than the levels found in leaves (Fig. 3 A, B). The variation was 2.6-fold for callus derived from the plant line containing the CaMV 35S promoter that was found to have a 12.3-fold variation amongst its leaves. The level of GUS expression from callus derived from a single CaMV 35S-containing plant line was 33-fold higher ($2,287 \pm 288$ nmoles 4-MU h^{-1} mg^{-1}) than the level (70 ± 19) from leaves of the same plant line. Callus derived from transgenic plants containing either the CaMV 35S or *UBQ3* promoters showed that the mean level of GUS activity was significantly higher (over 3×) for transgenic lines containing the CaMV 35S as compared to the *UBQ3* promoter (Table 2), and this confirmed the higher (2×) level of GUS activity in leaves of plants with the CaMV 35S promoter (Table 1).

CaMV 35S and duplicated CaMV 35S. A larger number of plants (three out of eight) expressed GUS activity exceeding 10 nmoles 4-MU h^{-1} mg^{-1} protein under the CaMV 35S promoter as compared to plants (one out of four) containing the duplicated CaMV 35S promoter (Fig. 3 C, E).

Five transgenic plants containing the CaMV 35S promoter were analyzed by Southern hybridization, and there was no apparent correlation between copy number or *bar-uidA* gene rearrangements and levels of GUS expression (Fig. 2 C, D). Exact copy number cannot be determined because the genome size of *Gladiolus* is unknown. Leaves from the three transgenic plants that expressed 173, 21, and 1 nmoles 4-MU h^{-1} mg^{-1} protein each contained an intact copy of the *bar-uidA* gene of similar band intensity (Fig. 2 C, lanes 4 and 5 and Fig. 2 D, lane 5). Leaves from two independently transformed plants that both showed very low levels of GUS, 1 nmole 4-MU h^{-1} mg^{-1} protein, contained an intact copy or multiple copies including three rearranged copies of *bar-uidA* (Fig. 2 D, lanes 4 and 5).

Transgenic plants containing the CaMV 35S promoter were initially identified by histochemical staining of callus regenerating small emerging shoots (Fig. 4 A). The regenerating callus pieces did not stain uniformly, and *uidA* was expressed most strongly in the small emerging shoots at the periphery of the callus. Transgenic plants with leaves that showed a strong positive reaction for GUS activity frequently exhibited variation in expression between leaves from an individual plant (Fig. 4 B). Some leaves stained dark blue and others were either white or showed a streaked pattern of expression. It was important to verify that the large variation in *uidA* expression between leaves of a plant was not due to a mixture of transformed and nontransformed leaves derived from a basal meristem that contained both transformed and nontransformed cells. Transgenic plants that exhibited variable staining between leaves were confirmed to be transformed by growth of the plant on MS basal salts medium supplemented with 4 mg l^{-1} PPT and Southern hybridization. Growth of *Gladiolus* plants on 4 mg l^{-1} PPT has been effective in selecting transformed plants from a mixed population of nontransformed and transformed plants (Kamo et al., 1995b).

There was no difference in tissue-specific patterns of *uidA* expression by all transgenic plants observed that contained *uidA* under either the CaMV 35S or duplicated CaMV 35S promoters as evaluated by histochemical staining as all transgenic plants containing either promoter exhibited the strongest *uidA* expression throughout the root, including the root tip, and either throughout the leaf including the vasculature, or only in the vasculature of leaves

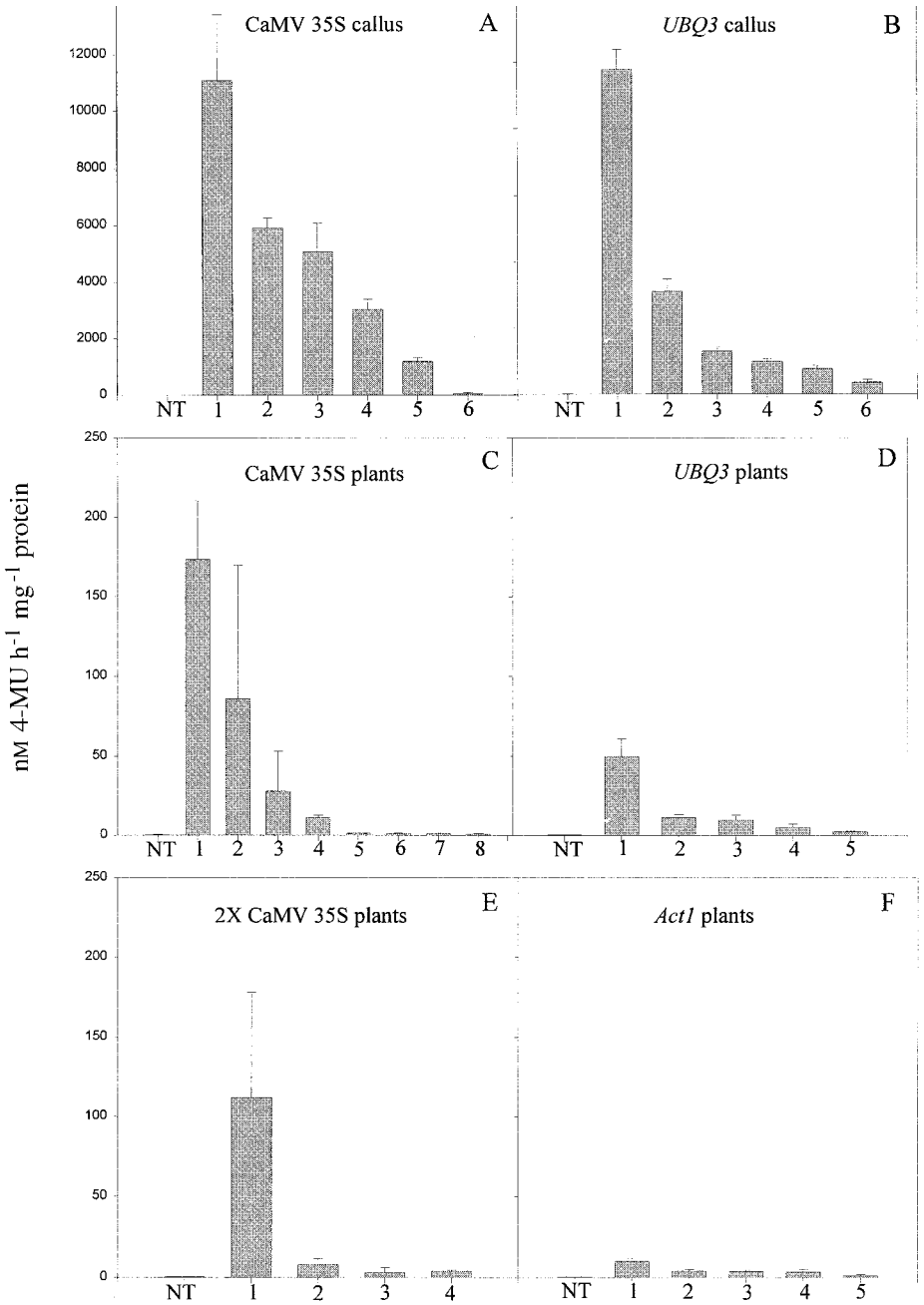

FIG. 3. Specific activity of β-glucuronidase in either transformed callus (A, B) or transgenic plants (C–F) of *Gladiolus* that contain *bar-uidA* under: A, the CaMV 35S; B, *UBQ3*; C, the CaMV 35S; D, duplicated CaMV 35S; E, *Act1*; and F, *UBQ3* promoters. Data for each independent transformant represent the mean ± standard error from three cloned plants derived from one independent transformant grown *in vitro*. Data for each transformed callus represent the mean ± standard error for three calluses grown in separate petri plates. The first sample shown in each graph represents a nontransformed (NT) plant or callus.

(Fig. 4 C). Leaves of young, 3–6-mm long shoots that emerged from callus showed less variation in expression than the older leaves (Fig. 4 D).

Act1 *promoter*. GUS activity was detected at a low level, ranging from 1.0 to 9.9 nmoles 4-MU h^{-1} mg^{-1} protein (Fig. 3 F), in leaves of all five transgenic plants of *Gladiolus* that contained *uidA* under the *Act1* promoter.

It was more difficult to regenerate transgenic plants containing *bar-uidA* under the *Act1* promoter compared to the CaMV 35S and *UBQ3* promoters because cells transformed under the *Act1* promoter continued to proliferate as callus rather than develop into plants when cultured on regeneration medium containing 1 or 3 mg l^{-1} bialaphos. Histochemical staining showed that *uidA* was expressed most strongly in young leaves, 1–2-cm long, and

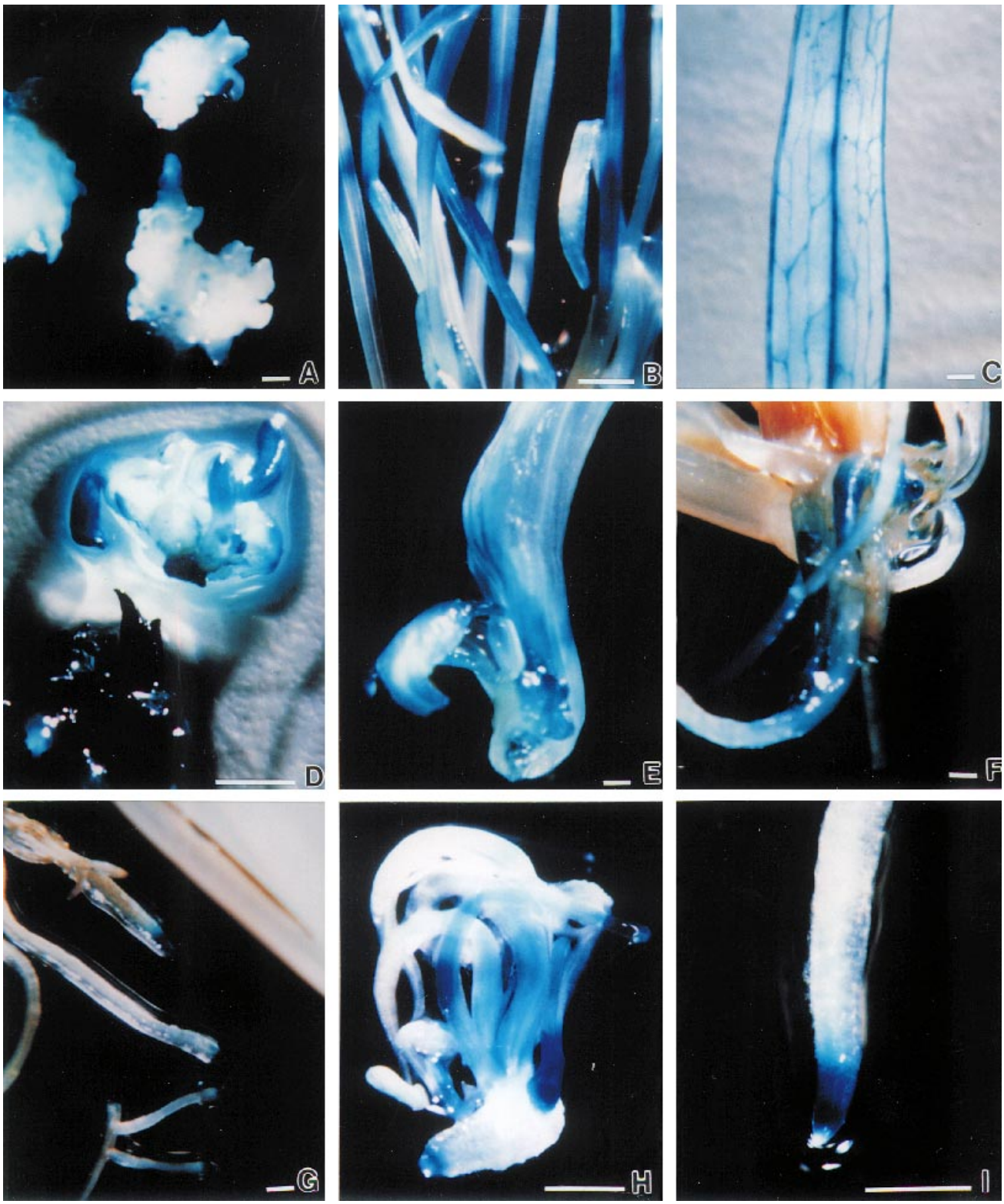

FIG. 4. Histochemical staining for *uidA* expression of *Gladiolus* plants transformed with *bar-uidA* under either the (A–D) CaMV 35S or duplicated CaMV 35S promoter, (E–G) *Act1* or (H, I) *UBQ3* promoters. A. Shoots, 2–3-mm long, emerging from callus. B. Leaves, approximately 5-cm long, from a single plant of *Gladiolus* grown *in vitro*. C. *UidA* expression in vasculature of a leaf. D. Shoots, 3–6-mm long, emerging from callus that developed from the base of transgenic plants grown *in vitro*. E. Young shoot, 2-cm long, that showed *uidA* expression. F. *UidA* expression by roots. G. *UidA* expression by root tips. H. Very young plant, 5-mm long, that contains the *UBQ3* promoter and exhibits *uidA* expression in its root tips and throughout its leaves. I. Root tips expressing *uidA* from the *UBQ3* promoter. Magnification bar represents 1 mm in all Figs., except B where it represents 25 mm.

TABLE 2

EFFECT OF THE CaMV 35S AND *UBQ3* PROMOTERS ON LEVELS OF GUS ACTIVITY IN CALLUS OF *GLADIOLUS* DERIVED FROM THE BASAL MERISTEM OF TRANSGENIC PLANTS. THE SPECIFIC ACTIVITY OF β-GLUCURONIDASE (MEAN ± STANDARD ERROR) FOR EACH PROMOTER IS CALCULATED FROM SIX TO SEVEN INDEPENDENTLY TRANSFORMED CALLUSES EACH SAMPLED THREE TIMES. ONLY CALLUSES THAT EXPRESSED GUS WERE INCLUDED IN THE DATA

		nmoles 4-MU h^{-1} mg^{-1} protein		
Promoter	Source	Highest level	Mean[a]	Relative GUS expression[b]
35S	CaMV	11,071.0	4,386.7 ± 954.5z	1.0
UBQ3	*Arabidopsis*	4,890.0	1,187.3 ± 353.9y	0.3

[a]Values followed by different letters are significantly different according to Kruskal–Wallis one-way analysis of variance at $P \leq 0.05$.
[b]Values represent relative GUS where 35S-GUS is set at 1.0.

throughout roots, particularly in root tips (Fig. 4 E–G). Older leaves, 5–6-cm long, did not exhibit visible *uidA* expression by histochemical staining although the fluorescent assay detected low levels of β-glucuronidase activity.

Ubiquitin promoters. Young callus-derived plants stained blue in the root tips and leaf bases (Fig. 4 H). Leaves, 5–6-cm long, from a *UBQ3*-containing plant exhibited a variable staining reaction between individual leaves. Some leaves stained intensely blue while other leaves were a pale blue color throughout their length. Strong *uidA* expression was observed in the root tips of older plants with 5–6-cm long leaves (Fig. 4 I).

Young leaves from *in vitro*-grown *Gladiolus* plants containing the *Ubi3* or *Ubi7* promoters never stained intensely blue but exhibited faint *uidA* expression localized in the vasculature, and the maximum level of GUS activity attained was 12.0 nmoles 4-MU h^{-1} mg^{-1} protein for 10 transgenic plants analyzed. Regenerable callus of *Gladiolus* transformed with the *Ubi7* promoter exhibited *uidA* expression. There was no GUS activity observed in cormels following histochemical staining, and the strongest GUS activity was in root tips which is consistent with *Ubi3* directing GUS expression in rapidly-dividing cells (Garbarino et al., 1992). Generally the level of GUS expression by leaves of *Gladiolus* plants transformed with the *UBQ3*, *Ubi3*, and *Ubi7* promoters declined as the plants aged.

Discussion

The promoters used for developing transgenic *Gladiolus* plants resulted in tissue-specific patterns of *uidA* expression consistent with that reported for cereal monocots. Leaves of transgenic rice and maize plants containing *uidA* under the CaMV 35S promoter exhibited the strongest levels of GUS activity localized in their vasculature (Battraw and Hall, 1990; Gordon-Kamm et al., 1990; Terada and Shimamoto, 1990) as was observed in transgenic *Gladiolus* plants. GUS activity was not uniform throughout the entire length of the leaf for both maize and *Gladiolus* (Gordon-Kamm et al., 1990). Register et al. (1994) found that leaves from individual plants of maize regenerated from a single callus piece exhibited variable GUS expression.

Primary and lateral roots of both transgenic rice and transgenic *Gladiolus* plants containing *uidA* under the CaMV 35S promoter exhibited the most intense staining in their root tips (Battraw and Hall, 1990). A larger number of transgenic *Gladiolus* plants with the CaMV 35S promoter showed GUS activity in roots (86%) compared to leaves (71%), as determined by histochemical staining.

In cereals the levels of transient GUS activity under *Act1* exceeded that of the CaMV 35S promoter by 19- and 21-fold in bombarded suspension cells of maize and polyethylene glycol-mediated transformed protoplasts of rice, respectively (McElroy et al., 1991; Zhang et al., 1991). Transient levels of GUS expression in suspension cells of *Gladiolus* bombarded with CaMV 35S were over four times higher than with the *Act1* promoter (Kamo et al., 1995a). This demonstrated a major difference in gene expression between cereals and a noncereal monocot that was confirmed by this study of transgenic *Gladiolus* plants.

Act1 directed GUS activity primarily in the rapidly-dividing cells of the root meristem and in very young leaves of *Gladiolus* (Fig. 4). High activity of the *Act1 5′* region in actively dividing tissues of transgenic *Gladiolus* is consistent with previous reports for rice, maize (Zhong et al., 1996), wheat (Nehra et al., 1994) and tritordeum (Barcelo et al., 1994). In roots of transgenic rice plants the most intense *uidA* staining was at the origin of secondary root formation, and root meristems also showed relatively strong GUS activity. *UidA* was expresssed in more cell types (leaf trichome, bundle sheath, root epidermal cells) and tissues (floral palea, stigma, stamen) of transgenic rice plants under the *Act1* promoter than the CaMV 35S promoter. It will be 3–4 yr before the transgenic *Gladiolus* plants flower when it can be determined if *Act1-uidA* is expressed in the reproductive tissues as occurs in rice.

The ubiquitin family consists of two classes of proteins, the polyubiquitin and the ubiquitin extension protein genes (Callis and Vierstra, 1989; Callis et al., 1990; Christensen et al., 1992). *Ubi7* and *UBQ3* are polyubiquitins isolated from potato (Garbarino et al., 1995) and *Arabidopsis* (Norris et al., 1993), respectively. *Ubi3* was isolated from potato (Garbarino and Belknap, 1994) and encodes a ubiquitin-ribosomal protein. Transgenic potato plants containing *uidA* under either the *Ubi3* or *Ubi7* promoter expressed high levels of *uidA* in young or senescent leaves and in tuber peel. *UidA* expression decreased as the leaves of potato aged although expression increased dramatically during leaf senescence (Garbarino and Belknap, 1994). The difference in GUS activity between *Ubi3*, *Ubi7* and *UBQ3* plants of *Gladiolus* cannot be directly compared because the *UBQ3* construct was a *bar-uidA* fusion gene whereas the *Ubi3* and *Ubi7* constructs were not fusion genes. It was anticipated that the *Ubi3* and *Ubi7* would result in fairly high levels of GUS activity because they were derived from a dicot and expressed high levels of GUS activity in leaves of transgenic potato plants; however, this did not occur in *Gladiolus* leaves as the maximum level of GUS was 12.0 nmoles 4-MU h^{-1} mg^{-1} protein.

This study determined that the cereal-derived promoter *Act1* and the dicot-associated promoters CaMV 35S and *UBQ3* will direct tissue-specific patterns of expression similarly between cereals and the noncereal monocot *Gladiolus*, although the levels of GUS activity differed between cereals and a noncereal monocot with these promoters. The level of GUS activity in the five *Gladiolus* plants containing *Act1* never reached the maximum level of GUS activity that could be expressed by a plant containing either of the two dicot-associated promoters, CaMV 35S or *UBQ3*.

Acknowledgment

The authors thank Anne O'Connor for technical assistance with DNA blots.

References

Barcelo, P.; Hagel, C.; Becker, D.; Martin, A.; Lorz, H. Transgenic cereal (tritordeum) plants obtained at high efficiency by microprojectile bombardment of inflorescence tissue. Plant J. 5:583–592; 1994.

Battraw, M. J.; Hall, T. C. Histochemical analysis of CaMV 35S promoter-β-glucuronidase gene expression in transgenic rice plants. Plant Mol. Biol. 15:527–538; 1990.

Callis, J.; Vierstra, R. D. Ubiquitin and ubiquitin genes in higher plants. Oxford Surv. Plant Mol. Cell Biol. 6:1–30; 1989.

Callis, J.; Raasch, J. A.; Vierstra, R. D. Ubiquitin extension proteins of *Arabidopsis thaliana*. J. Biol. Chem. 265:12485–12493; 1990.

Cao, J.; Duan, X.; McElroy, D.; Wu, R. Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. Plant Cell Rep. 11:586–591; 1992.

Christensen, A. H.; Sharrock, R. A.; Quail, P. H. Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript slicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol. Biol. 18:675–689; 1992.

Dellaporta, S.; Wood, J.; Hicks, J. A plant DNA minipreparation: version II. Plant Mol. Biol. Rep. 1:19–21; 1983.

Garbarino, J. E.; Rockhold, D. R.; Belknap, W. R. Expression of stress-responsive ubiquitin genes in potato tubers. Plant Mol. Biol. 20:235–244; 1992.

Garbarino, J. E.; Belknap, W. R. Isolation of a ubiquitin-ribosomal protein gene (*ubi3*) from potato and expression of its promoter in transgenic plants. Plant Mol. Biol. 24:119–127; 1994.

Garbarino, J. E.; Oosumi, T.; Belknap, W. R. Isolation of a polyubiquitin promoter and its expression in transgenic potato plants. Plant Physiol. 109:1371–1378; 1995.

Gordon-Kamm, W. J.; Spencer, T. M.; Mangano, M. L.; Adams,T. R.; Daines, R. J.; Start, W. G.; O'Brien, J. V.; Chambers, S. A.; Adams, W. R. Jr.; Willett, N. G.; Rice, T. B.; Mackey, C. J.; Krueger,W.; Kausch, A. P.; Lemaux, P. Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell 2:603–618; 1990.

Jefferson, R. A.; Kavanagh, T. A.; Bevan, M. W. GUS-fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3901–3907; 1987.

Kamo, K.; Blowers, A.; Smith, F.; Van Eck, J.; Lawson, R. Stable transformation of *Gladiolus* using suspension cells and callus. J. Am. Soc. Hort. Sci. 120:347–352; 1995a.

Kamo, K.; Blowers, A.; Smith, F.; Van Eck, J. Stable transformation of *Gladiolus* by particle gun bombardment of cormels. Plant Sci. 110:105–111; 1995b.

Maniatis, R.; Fritsch, E .F.; Sambrook, J. Molecular cloning. A laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1982:368–369.

McElroy, D.; Blowers, A. D.; Jenes, B.; Wu, R. Construction of expression vectors based on the rice actin (*Act1*) 5′ region for use in monocot transformation. Mol. Gen. Genet. 23:150–160; 1991.

McElroy, D.; Chamberlain, D. A.; Moon, E.; Wilson, K. J. Development of *gusA* reporter gene constructs for cereal transformation: Availability of plant transformation vectors from the CAMBIA Molecular Genetic Resource Service. Mol. Breed. 1:27–37; 1995.

Murakami, T.; Anzai, H.; Imai, S.; Satoh, A.; Nagaoka, K.; Thompson, C. J. The bialaphos biosynthetic genes of *Streptomyces hygroscropicus*: Molecular cloning and characterization of the gene cluster. Mol. Gen. Genet. 205:42–50; 1986.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15:473–497; 1962.

Nehra, N. S.; Chibbar, R. N.; Leung, N.; Caswell, K.; Mallard, C.; Steinhauer, L.; Baga, M.; Kartha, K. K. Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene constructs. Plant J. 5:285–297; 1994.

Norris, S. R.; Meyer, S. E.; Callis, J. The intron of *Arabidopsis thaliana* polyubiquitin genes is conserved in location and is a quantitative determinant of chimeric gene expression. Plant Mol. Biol. 21:895–906; 1993.

Odell, J. R.; Nagy, F.; Chua, N. Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313:170–173; 1985.

Register III, J. C.; Peterson, D. J.; Bell, P. J.; Bullock, W. P.; Evans, I. J.; Frame, B.; Greenland, A. J.; Higgs, N. S.; Jepson, I.; Jiao, S.; Lewnau, C. J.; Stillick, J. M.; Wilson, H. M. Structure and function of selectable and non-selectable transgenes in maize after introduction by particle bombardment. Plant Mol. Biol. 25:951–961; 1994.

Russell, J. A.; Roy, M. K.; Sanford, J. C. Physical trauma and tungsten toxicity reduce the efficiency of biolistic transformation. Plant Physiol. 98:1050–1056; 1992.

Sanford, J. C.; Smith, F. D.; Russell, J. A. Optimizing the biolistic process for different biological applications. Methods Enzymol. 217:483–510; 1993.

Schledzewski, K.; Mendel, R. R. Quantitative transient gene expression: comparison of the promoters for maize polyubiquitin 1, rice actin 1, maize-derived emu and CaMV 35S in cells of barley, maize and tobacco. Trans. Res. 3:249–255; 1994.

Shimamoto, K. Gene expression in transgenic monocots. Curr. Opin. Biotech. 5:158–162; 1995.

Spencer, T. M.; O'Brien, J. V.; Start, W. G.; Adams, T. R.; Gordon-Kamm, W. J.; Lemaux, P. G. Segregation of transgenes in maize. Plant Mol. Biol. 18:201–210; 1992.

Terada, R.; Shimamoto, K. Expression of CaMV 35S-GUS gene in transgenic rice plants. Mol. Gen. Genet. 220:389–392; 1990.

Wehrmann, A.; Van Vilet, A.; Opsomer, C.; Botterman, J.; Schulz, A. The similarities of *bar* and *pat* gene products make them equally applicable for plant engineers. Nature Biotechnol. 14:1274–1278; 1996.

Wilmink, A.; Van de Ven, B. C. E.; Dons, J. J. M. Activity of constituitive promoters in various species of the Liliaceae. Plant Mol. Biol. 28:949–955; 1995.

Zhang, W.; McElroy, D.; Wu, R. Analysis of the rice Act1 5′ region activity in transgenic rice plants. Plant Cell 3:1155–1165; 1991.

Zhong, H.; Zhang, S.; Warkentin, D.; Sun, B.; Wu, T.; Wu, R.; Sticklen, M. B. Analysis of the functional activity of the 1.4 kb 5′-region of the rice actin 1 gene in stable transgenic plants of maize (*Zea mays* L.). Plant Sci. 116:73–84; 1996.